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Development of a *Schistosoma mansoni* shotgun O-glycan microarray and application to the discovery of new antigenic schistosome glycan motifs

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ABSTRACT

Upon infection with *Schistosoma*, antibody responses are mounted that are largely directed against glycans. Over the last few years significant progress has been made in characterising the antigenic properties of N-glycans of *Schistosoma mansoni*. Despite also being abundantly expressed by schistosomes, much less is understood about O-glycans and antibody responses to these have not yet been systematically analysed. Antibody binding to schistosome glycans can be analysed efficiently and quantitatively using glycan microarrays, but O-glycan array construction and exploration is lagging behind because no universal O-glycanase is available, and release of O-glycans has been dependent on chemical methods. Recently, a modified hydrazinolysis method has been developed that allows the release of O-glycans with free reducing termini and limited degradation, and we applied this method to obtain O-glycans from different *S. mansoni* life stages. Two-dimensional HPLC separation of 2-aminobenzoic acid-labelled O-glycans generated 362 O-glycan-containing fractions that were printed on an epoxide-modified glass slide, thereby generating the first shotgun O-glycan microarray containing naturally occurring schistosome O-glycans. Monoclonal antibodies and mass spectrometry showed that the O-glycan microarray contains well-known antigenic glycan motifs as well as numerous other, potentially novel, antibody targets. Incubations of the microarrays with sera from *Schistosoma*-infected humans showed substantial antibody responses to O-glycans in addition to those observed to the previously investigated N- and glycosphingolipid glycans. This underlines the importance of the inclusion of these often schistosome-specific O-glycans in glycan antigen studies and indicates that O-glycans contain novel antigenic motifs that have potential for use in diagnostic methods and studies aiming at the discovery of vaccine targets.

1. Introduction

Schistosomiasis (bilharzia) is a chronic and potentially deadly parasitic disease. It is a major public health burden in (sub)tropical areas with at least 230 million people being affected and many more at risk of being infected with schistosomes (Gryseels et al., 2006; Steinmann et al., 2006; Vos et al., 2012). Schistosomes have a complex life-cycle with larval, adult worm and egg stages interacting with the human host, each playing a role in immunology, immunopathology and maintenance of infection. Although a schistosome infection can be treated effectively with Praziquantel (Fenwick and Webster, 2006; Gray et al., 2011), this does not prevent reinfection, emphasising the need for the development of a prophylactic vaccine inducing protection against schistosomiasis. Natural immunity to *Schistosoma* does occur, but it takes many years of infection to develop, is gradually acquired with age and involves several immunological parameters such as T cell and specific antibody responses (Butterworth et al., 1988; Khalife et al., 1989; Leenstra et al., 2006; Vereecken et al., 2007).

Antibody responses in schistosomiasis have mainly been studied using crude antigens or (recombinant) proteins. The majority of antibodies generated during *Schistosoma* infection are, however, directed against the highly abundant and immunogenic parasite glycans (Eberl et al., 2001; Hokke and Deelder, 2001; Naus et al., 2003b; Nyame et al., 2003; Hokke et al., 2007a,b; Kariuki et al.,

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2008). *Schistosoma* life stages each express a different glycan repertoire (Nyame et al., 2004; Hokke and Yazdanbakhsh, 2005; Hokke et al., 2007a). Elaborate studies on the glycome of the different *Schistosoma* life stages have indicated that hundreds of different glycan structures are present within the N- and O-linked glycans and the glycosphingolipid glycans (GSL-glycans) (Hokke et al., 2007a). Recently we showed, by shotgun glycan microarray, that anti-glycan antibodies are induced against many N-linked and GSL-glycans in schistosome-infected individuals and that the shotgun glycan microarray is a valuable tool in defining antigenic glycans and glycan recognition profiles that can discriminate groups within a serum cohort (Van Diepen et al., 2012a).

To date, schistosome-related glycan microarray studies have mainly incorporated synthetic glycans, or N-glycans and GSL-glycans that can be enzymatically derived from natural sources (Van Diepen et al., 2012a; Mandalasi et al., 2013; McWilliam et al., 2013; Luyai et al., 2014). Convenient methods to release and recover intact reducing O-glycans such that they can be reacted with a label or linker molecule and printed on a covalent microarray are not yet available. However, O-glycans are highly abundant in schistosome cercariae and eggs (Khoo et al., 1995, 1997; Huang et al., 2001; Jang-Lee et al., 2007) and antibody responses to these glycans are likely to occur. Since some terminal antigenic glycan elements are shared between different types of glycans it is likely that subsets of anti-O-glycan antibodies will be directed against antigenic glycan motifs that also occur as part of N- or glycolipid glycans, but antibody responses to O-glycan-specific motifs are also expected to be present. Unlike N-glycans, biosynthesis of O-glycans occurs by sequential addition of single glycosyl residues rather than via the initial transfer of a common conserved lipid-linked precursor to the growing protein backbone. As a consequence, O-glycans can display multiple core structures (Brockhausen et al., 2009). For *Schistosoma*, three core structures have been defined to date (Khoo et al., 1997; Huang et al., 2001; Jang-Lee et al., 2007). The majority of O-glycans expressed by cercarial secretions contain the Galβ1-3(Galβ1-6)GalNAc core (*Schistosoma*-specific) which can be modified by additional β1-6Gal to Gal, but structures with Galβ1-3(GlcNAcβ1-6)GalNAc core (mucin-type 2) have also been observed (Jang-Lee et al., 2007). The cercarial glycocalyx has been shown to specifically carry complex O-glycans with repeating units of unique multi-fucosylated -3GalNAcβ1-4GlcNAcβ1-4Galα1- motifs (Khoo et al., 1995), but the overall major terminal motifs expressed by cercarial O-glycans are Galβ1-4(Fucα1-3)GlcNAc (LeX) and tandem LeX (Jang-Lee et al., 2007). In adult worms, O-glycans could not be directly detected by MS (Wuhrer et al., 2006) but it has been shown previously by other methods that adult worms excrete circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) that carry large O-glycans with repeating LeX and GlcA-substituted GalNAc motifs, respectively (Bergwerff et al., 1994; van Dam et al., 1994). The egg-stage O-glycans are characterised by a multitude of different terminal motifs. In contrast to cercariae and schistosomula, the most predominant core motif is the mucin type-2 core and more structures with higher masses are expressed. Similar to cercariae, relatively high levels of LeX as well as (multi-)fucosylated GalNAcβ1-4GlcNAc (LDN) elements are expressed (Khoo et al., 1997; Jang-Lee et al., 2007; Robijn et al., 2007).

In view of the unique core structures of schistosome O-glycans and the expected occurrence of antigenic glycans motifs not present in other glycan classes, we set out to develop a schistosome shotgun O-glycan microarray. We applied a modified hydrazinolysis method that allows the release of O-glycans with free reducing termini in high yields with reduced levels of degradation products (Kozak et al., 2012). O-glycans from cercariae, adult worms and eggs were released, labelled with 2-aminobenzoic acid (2-AA) and fractionated by two-dimensional HPLC. These were then printed on an epoxide-modified glass slide to generate the first *Schistosoma* O-glycan microarray. This new O-glycan array was tested and compared with existing N- and GSL-glycan arrays using anti-glycan monoclonal antibodies (mAbs) and infection sera, revealing the occurrence of a number of antigenic O-glycans to which substantial antibody responses are raised in schistosome infection.

2. Materials and methods

2.1. Sera and ethics statement

Human sera were obtained from *Schistosoma mansoni*-infected individuals living in the Piida community, Butiaba, which is situated on the shore of Lake Albert in Uganda where *S. mansoni* is endemic. Ethical approval for the Piida study was obtained from the Uganda National Council for Science and Technology (UNCST) and cleared by the Office of the President. The study was also supported by the Cambridge Local Research Ethics Committee, UK. Prior to enrolment, the study was explained to each selected adult or parent/guardian of each child selected for the study and verbal consent was obtained. The study design, epidemiology and sample collection have been described in detail previously (Naus et al., 2003a). The child and adult sera used in this study were non-randomly selected from the original Piida cohort study, matched for infection intensity and balanced for sex as described previously (Van Diepen et al., 2012a).

2.2. Materials

Mouse mAbs reactive to schistosome glycans were generated from *S. mansoni*-infected mice and previously characterised by screening against a small panel of synthetic neoglycoconjugates (van Remoortere et al., 2000; Robijn et al., 2005). Included in the present study were mAbs that bind to GalNAcβ1-4GlcNAc (LacdiNAc, LDN, mAb 273-3F2), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F, mAb 114-4E8-A), Fucα1-3GalNAcβ1-4GlcNAc, (F-LDN, mAb 291-5D5), and Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX, mAb 291-4D10) (van Remoortere et al., 2000; Robijn et al., 2005; Wuhrer et al., 2006).*Schistosoma mansoni* adult worms, cercariae and eggs were obtained as reported previously (Robijn et al., 2005). BSA- and NH_2-linked synthetic oligosaccharide conjugates were synthesised as previously described (Vermeer et al., 2000, 2003; van Roon et al., 2005; de Boer et al., 2007, 2008). Cy3 conjugated goat anti-human IgG (Fc-specific), BSA and ethanolamine were from Sigma (Zwijndrecht, The Netherlands). Alexa Fluor 647 conjugated goat anti-human IgM (μ chain specific), Alexa Fluor 647 conjugated rabbit anti-mouse IgG (H+L), and Alexa Fluor conjugated goat anti-mouse IgM (μ chain specific) were from Invitrogen (Breda, The Netherlands).

2.3. O-glycan release

O-glycans were released as described previously (Kozak et al., 2012). Briefly, *S. mansoni* adult worms, cercarial and egg protein extracts were washed three times with 0.1% trifluoroacetic acid (TFA) using centrifugal filtration tubes with a molecular weight cut-off of 10 kDa and dried down by vacuum centrifugation. The O-glycans were released by addition of hydrazine and incubation at 60 °C for 6 h. Hydrazine was removed by centrifugal evaporation. Samples were then placed on ice and re-*N*-acetylated by the addition of 200 μl of 0.1 M sodium bicarbonate and 21 μl of acetic anhydride. Samples were vortexed and incubated on ice for 10 min. A further aliquot of acetic anhydride (21 μl) was added to each sample, followed by vortexing and incubation at room temperature for 50 min. Released O-glycans were cleaned up by passing them

through Ludger-Clean CEX cartridges (Ludger Ltd., Oxfordshire, UK) and were dried by vacuum centrifugation prior to further use.

2.4. N-glycan and GSL-glycan release

N-glycans and GSL-glycans were released as described previously (Van Diepen et al., 2012a). *Schistosoma mansoni* male and female worms, cercariae and eggs were homogenised in water (4 ml per g wet weight) and methanol and chloroform were added sequentially (7 and 13 volumes, respectively). The upper phase contained the GSL and the pellet the (glyco)proteins. Glycans were released from the different preparations of *S. mansoni* glycolipids and glycoproteins by glycoceramidase and PNGase F treatment, respectively.

2.5. Labelling and fractionation of released glycans

Released O-, N-, and GSL-glycans were labelled with 2-AA as described previously (Ruhaak et al., 2010) and fractionated by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection on a TSK gel Amide-80 column (4.6 mm inner diameter (ID) × 15.0 cm, particle size 3 μm, Tosoh Bioscience, Germany). Eluent A consisted of 100% acetonitrile (ACN), Eluent B consisted of 50 mM ammonium formate pH 4.4. A linear gradient from 22.4% to 54.4% eluent B in 40 min was applied at a flow rate of 1 ml/min. Fluorescence detection was performed at λ_{ex}–λ_{em} 360–425 nm. HILIC peak fractions were collected and dried by centrifugal evaporation, and subsequently sub-fractionated in a second dimension using reversed-phase (RP)-HPLC on a Superspher 100 RP-18 column (Merck Millipore, Germany). Eluent A consisted of 0.1% formic acid; eluent B consisted of 95% ACN with 0.1% formic acid. A linear gradient from 5% to 50% eluent B was applied at a flow rate of 200 μl/min. Fluorescence detection was performed at λ_{ex}–λ_{em} 360–425 nm and all peak fractions were collected.

2.6. Mass spectrometry

Glycan samples were analysed by MALDI-TOF-MS with Ultraflex II and Ultraflextreme mass spectrometers (Bruker Daltonics, Bremen, Germany) in the negative ion reflectron mode using 2,5-dihydroxybenzoic acid (DHB, Bruker Daltonics) (20 mg/ml in 30% ACN) as matrix. Glycopeakfinder (http://www.glyco-peakfinder.org) was used as an aid to define glycan compositions.

2.7. Glycan microarray construction

Glycan-containing HPLC fractions, (synthetic) glycoconjugates, and (glyco)proteins were dissolved in 20 μl of 1× spotting buffer (Nexterion Spot, Schott Nexterion, Germany) with 10% DMSO in 384-well small volume, deep well polypropylene plates (Greiner Bio-One, Germany). Two types of arrays were printed, one containing the N-glycans and GSL-glycans, and one containing the O-glycans. For the N- and GSL-glycan array 389 samples (115 from adult worms, 127 from cercaria, 110 from eggs and 37 from cercarial glycolipids) were printed. For the O-glycan array 362 samples (79 from adult worms, 137 from cercariae and 146 from eggs), were printed. Both arrays also included synthetic neoglycoconjugates (BSA-LeX, BSA-LDN, BSA-LDN-F, BSA-F-LDN, BSA-F-LDN-F) as well as (glyco)protein extracts from *S. mansoni* (adult worm antigen (AWA), TCA-precipitate of AWA (AWA-TCA), soluble egg antigen (SEA), excretory/secretory (glyco)proteins from eggs (ES), and cercarial antigen (CA)) as controls for binding of the mAbs.

Glycan amounts were estimated based on peak height in the HPLC and each sample was printed in triplicate at different concentrations (three-fold dilutions, starting from 10 μM or as high as the sample amounts allowed) on epoxysilane-coated glass slides (Slide E, Schott, Nexterion, Germany) by contact printing using the Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI, USA) equipped with SMP3 pins with uptake channels that deposit 0.7 nl at each contact. Each array was printed three times on each glass slide. Dot spacing was 290 μm (X) and 245 μm (Y), and array spacing was 6000 μm. Printed slides were incubated overnight at room temperature at sufficient humidity to prevent drying of the spots and to allow covalent binding of printed 2-AA labelled glycans and glycoconjugates to the epoxysilane coating via reaction with primary or secondary amines (de Boer et al., 2007).

2.8. Binding assay

Microarray slides were covered with a hand-cut silicone gasket creating barriers to separate the three printed arrays and to keep wash and incubation solutions within the individual array areas. To remove unbound compounds, the arrays were rinsed with 1 ml of PBS. Remaining active epoxysilane groups were blocked with 2% BSA, 50 mM ethanolamine in PBS for 60 min at room temperature while shaking. Subsequently, the slides were rinsed with PBS. Each microarray was incubated with mAb or serum diluted in PBS-0.01% Tween20 with 1% BSA (mAbs diluted 1:200 or 1:1000; serum diluted 1:100) for 60 min at room temperature while shaking. After washing with successive rinses of PBS-0.05% Tween20 and PBS, the slides were incubated with fluorescently labelled anti-IgG and/or anti-IgM (diluted 1:1000 in PBS-0.01 Tween20) for 30 min at room temperature while shaking and protected from exposure to light. After a final rinse with PBS-0.05% Tween20, PBS and water, the slides were dried and kept in the dark until scanning.

2.9. Scanning and data analysis

A G2565BA scanner (Agilent Technologies, Santa Clara, CA, USA) was used to scan the slides for fluorescence at 10 μm resolution using two lasers (532 nm and 633 nm). At these wavelengths the 2-AA label does not fluoresce. Data and image analysis were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA). Spots were aligned and re-sized using round features with no composite pixel intensity (CPI) threshold. Background-subtracted median intensities were averaged and processed as described previously (Oyelaran et al., 2009; Van Diepen et al., 2012a) and median values of negative controls included on each array were subtracted.

3. Results

3.1. Generation of a shotgun O-glycan microarray

O-glycans were obtained from different life stages (cercariae, adult worms and eggs) of *S. mansoni* by hydrazinolysis, labelled with 2-AA and purified. These samples were analysed by MALDI-TOF-MS to determine their glycan contents. A broad range of O-glycan compositions were present in the cercarial- and egg-derived samples and, although there was some overlap in compositions, the majority of compositions and proposed structures differed between these sources. (Fig. 1A, C; Table 1). These samples were fractionated by HILIC and a total number of 23 cercarial and 36 egg fractions were collected (Fig. 1B, D). For the adult worm-derived O-glycan sample, no glycans could be detected by MALDI-TOF-MS but the HILIC profile showed 28 low intensity peaks that were collected as fractions (data not shown). For all

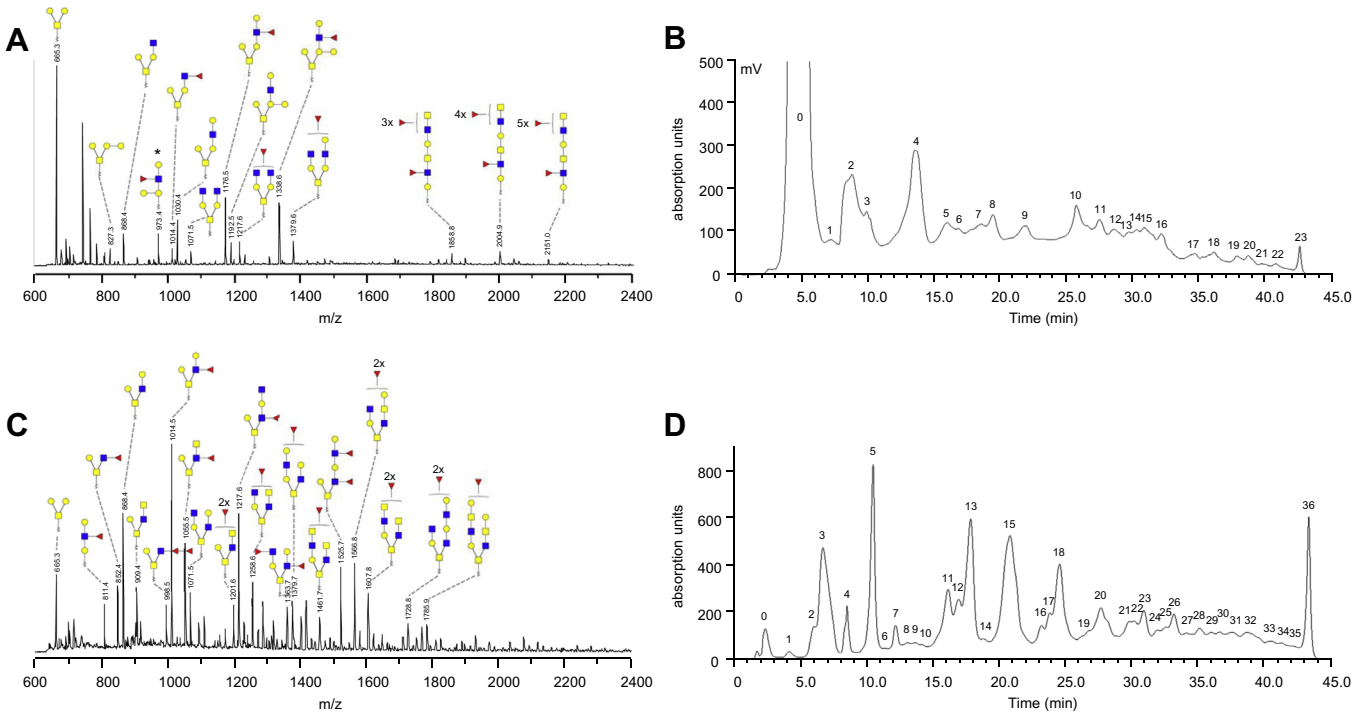

Fig. 1. Negative-ion MALDI-TOF-MS spectra (A, C) and hydrophilic interaction liquid chromatography chromatograms with fluorescence detection (B, D) of *Schistosoma mansoni* cercarial- (A, B) and egg-derived (C, D) 2-aminobenzoic acid-labelled O-glycan pools. All MALDI-TOF-MS signals are labelled with monoisotopic masses, structures are shown for peaks with signal/noise higher than 10 and are based on data from the literature (Khoo et al., 1995; Huang et al., 2001; Jang-Lee et al., 2007) and our unpublished MS/MS data. HILIC chromatograms with numbers indicating the fractions that were collected. Blue square, *N*-acetylglucosamine; yellow square, *N*-acetylgalactosamine; yellow circle, galactose; red triangle, fucose. The asterisk indicates a 2-aminobenzoic acid-labelled peeling product. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

three samples, each collected HILIC fraction was further fractionated in a second dimension using RP-HPLC. All collected RP fractions were then analysed by MALDI-TOF-MS. Glycans were detected in 114 cercarial, 50 adult worm, and 110 egg RP-HPLC fractions with a total number of 178, 46 and 170 different glycan compositions, respectively. The adult worm-derived fractions contained only very low amounts of O-glycans based on peak heights in HPLC and in MALDI-TOF-MS. Only the fractions containing glycans identified by MS were used to generate O-glycan microarrays. These arrays were probed with anti-glycan mAbs and human sera to uncover antigenic O-glycan structures. For comparison, we used the previously generated shotgun glycan microarray containing N-glycans and lipid glycans derived from *S. mansoni* cercariae, adult worms and eggs (Van Diepen et al., 2012a).

3.2. mAb binding

To test the functionality of the array, we used four anti-glycan mAbs reactive with glycan structures expressed by different life stages of *S. mansoni* as previously determined by immunofluorescence microscopy (Table 2), and analysed these on the new shotgun O-glycan microarray as well as the previously generated shotgun N- and GSL-glycan microarray for comparison. For all mAbs tested, the binding to crude aqueous extracts of adult worms, cercariae and eggs was analysed and glycan specificity was confirmed by binding to synthetic conjugate controls printed on the arrays (Table 2).mAb 291-4D10-A (anti-LeX) bound to glycans expressed by worms (gut, parenchyma and tegument) and eggs (shell and excreted antigen), while for cercariae only the oral sucker was stained in the immunofluorescence assay (Table 2). Hardly any binding to cercarial-derived O-glycans was observed, while responses against egg-derived O-glycans were very high, especially for the more complex O-glycans originating from late HILIC fractions (Fig. 2A). This indicates that many of the egg-derived O-glycans contain LeX elements to which the antibody bound, while this was much less for the cercarial-derived O-glycans. With respect to the N-glycan microarray, we observed binding to many of the cercarial GSL-glycans and to only a few worm, cercarial and egg N-glycans (Fig. 2A).

The mAbs 273-3F2 (anti-LDN) and 114-4E8 (anti-LDN-F) both bound to structures expressed by worms (parenchyma and tegument) and eggs (shell and miracidia), while binding to cercariae was lacking (Table 2). In addition, 114-4E8 showed binding to structures expressed in the worm gut, whereas 273-3F2 bound to excreted egg antigen. The mAb 273-3F2 showed no binding to any of the O-glycans printed, indicating that none of those contained the non-substituted LDN element, relative abundance of LDN was too low to be detected by the antibody, or the antibody only binds to LDN in a specific structural context (Fig. 2B). mAb 114-4E8, on the other hand, did show binding to some of the egg O-glycans but binding to cercarial O-glycans was virtually absent (Fig. 2C). This shows that some of the egg O-glycans contain LDN-F, while cercarial O-glycans do not. In addition to egg glycolipids, mainly egg glycoproteins expressing LDN-F have previously been shown to be the major targets of this mAb on IFA and Western blots (Nyame et al., 2003; Robijn et al., 2005). The major difference between the two antibodies is that 114-4E8 showed binding to egg O- and N-glycans while 273-3F2 only bound N-glycans (Fig. 2B, C). The binding properties of mAb 273-3F2 have not been described before, but Nyame et al. (2003) described another anti-LDN antibody and showed that it bound more abundantly to egg glycoproteins than to glycoproteins from cercariae (Nyame et al., 2003). Also the anti-LDN antibody that was used in our study showed binding to egg-derived N-glycans

Table 1
Glycan masses and compositions in released O-glycan pools from different life stages of *Schistosoma mansoni*.

Registered *m/z*[a]	Glycan composition[b]	Core[c]	Proposed glycan motifs[d]	Registered *m/z*[a]	Glycan composition[b]	Core[c]	Proposed glycan motifs[d]
Cercarial-derived O-glycan pool							
665.287	H2N1-AA	II	LN	1338.558	F1H4N2-AA	III	LeX, β1-6Gal, (F_{0-1})Gn
811.342	F1H2N1-AA	V	LeX	1347.603	F3H1N3-AA	I	LDN(F_3), F_{1-3}Gn
827.342	H3N1-AA	III,V	LN, β1-6Gal	1687.740	F2H4N3-AA	II	LeX, diLeX
852.370	F1H1N2-AA	I	FGn	1696.784	F4H1N4-AA	I	LDN(F_{1-4}), (F_{0-3})Gn
868.371	H2N2-AA	I,II	LN, Gn	1712.782	F3H2N4-AA	IV	LDN(F_{0-3})
973.408	F1H3N1-AA	V	LeX, β1-6Gal	1842.844	H3N6-AA	IV	LDN
998.452	F2H1N2-AA	I	F_2Gn		F5H1N4-AA	I	LDN(F_{2-5}), (F_{0-3})Gn
1014.439	F1H2N2-AA	I,II	LeX, FGn	1858.837	F4H2N4-AA	I	LDN(F_{1-4})
1030.433	H3N2-AA	II	LN	1988.920	F1H3N6-AA	IV	LDN(F_{0-1})
1055.477	F1H1N3-AA	I	LDN(F_{0-1}), (F_{0-1})Gn		F6H1N4-AA	I	LDN(F_{3-6}), (F_{0-3})Gn
1144.514	F3H1N2-AA	I	F_3Gn	2004.895	F5H2N4-AA	IV	LDN(F_{2-5})
1176.496	F1H3N2-AA	II	LeX	2061.949	F4H2N5-AA	I	Gal-LDN(F_{0-4}), LDN(F_{0-4})
1192.496	H4N2-AA	III	LN, β1-6Gal, Gn	2150.972	F6H2N4-AA	IV	LDN(F_{3-6})
1201.537	F2H1N3-AA	I	LDN(F_{0-2}), (F_{0-2})Gn	2208.012	F5H2N5-AA	I	Gal-LDN(F_{0-5}), LDN(F_{0-5})
Egg-derived O-glycan pool							
665.328	H2N1-AA	II	LN	1493.755	F4H1N3-AA	I	LDN(F_4), (F_2)Gn
811.401	F1H2N1-AA	V	LeX	1525.742	F2H3N3-AA	I,II	LeX, di-LeX, FGn
852.435	F1H1N2-AA	I	FGn	1550.778	F3H1N4-AA	I	LDN(F_{0-3}), (F_{0-2})Gn
868.426	H2N2-AA	I,II	LN, Gn	1566.770	F2H2N4-AA	I	Gal-LDN(F_{0-2}), (F_{0-2})Gn
909.449	H1N3-AA	I	LDN, Gn	1582.775	F1H3N4-AA	I,II	LeX, (F)Gn, LN
998.504	F2H1N2-AA	I	F_2Gn	1607.806	F2H1N5-AA	I	LDN(F_{0-2})
1014.498	F1H2N2-AA	I,II	LeX, FGn	1623.811	F1H2N5-AA	I	Gal-LDN(F_{0-1}), LDN(F_{0-1})
1055.529	F1H1N3-AA	I	LDN(F_{0-1}), (F_{0-1})Gn	1639.816	H3N5-AA	I	Gal-LDN
1071.526	H2N3-AA	I,II	Gal-LDN, Gn		F5H1N3-AA	I	LDN(F_5)
1112.552	H1N4-AA	I	LDN, Gn	1712.862	F3H2N4-AA	I	Gal-LDN(F_{0-3}), (F_{0-2})Gn
1160.568	F2H2N2-AA	V	Gal-LDN(F_2)	1728.852	F2H3N4-AA	I,II	LeX, diLeX, (F)Gn, LN
1176.562	F1H3N2-AA	II	LeX	1744.844	F1H4N4-AA	I,II	LeX, LN, (F)Gn
1201.600	F2H1N3-AA	I	LDN(F_2), (F_{0-2})Gn	1753.889	F3H1N5-AA	I	LDN(F_{0-3})
1217.594	F1H2N3-AA	I,II	LeX, LN, (F_{0-1})Gn	1769.886	F2H2N5-AA	I	Gal-LDN(F_{0-2}), LDN(F_{0-2})
1233.596	H3N3-AA	I,II	LN, Gn	1785.900	F1H3N5-AA	I	Gal-LDN(F_{0-1})
1258.622	F1H1N4-AA	I	LDN(F_{0-1}), (F_{0-1})Gn	1874.932	F3H3N4-AA	I,II	LeX, diLeX, FGn
1274.630	H2N4-AA	I	Gal-LDN, Gn	1890.934	F2H4N4-AA	I,II	LeX, diLeX, LN, (F)Gn
1290.647	F4H1N2-AA	V	LDN(F_4)	1899.953	F4H1N5-AA	I	LDN(F_{0-4})
1315.660	H1N5-AA	I	LDN	1931.959	F2H3N5-AA	I	Gal-LDN(F_{0-2})
1322.641	F2H3N2-AA	V	diLeX	1973.019	F2H2N6-AA	I	LDN(F_{0-2}), (F)Gn
1347.669	F3H1N3-AA	I	LDN(F_3), (F_{0-3})Gn	1989.032	F1H3N6-AA	I	Gal-LDN(F_{0-1}), (F)Gn
1363.673	F2H2N3-AA	I,II	LeX, FGn	2037.004	F3H4N4-AA	I,II	LeX, diLeX, tri-LeX, FGn
1379.671	F1H3N3-AA	I,II	LeX, LN, (F_{0-1})Gn	2078.045	F3H3N5-AA	I	Gal-LDN(F_{0-3})
1404.702	F2H1N4-AA	I	LDN(F_{0-2}), (F_{0-2})Gn	2094.036	F2H4N5-AA	I,II	LeX, di-LeX, (F)Gn
1420.700	F1H2N4-AA	I	Gal-LDN(F_{0-1}), (F_{0-1})Gn	2119.097	F3H2N6-AA	I	LDN(F_{0-3}), (F_{0-2})Gn
1436.693	H3N4-AA	I,II	LN, Gn	2135.097	F2H3N6-AA	I	Gal-LDN(F_{0-2}), (F_{0-2})Gn
1461.725	F1H1N5-AA	I	LDN(F_{0-1})	2240.139	F3H4N5-AA	I	LeX, di-LeX, LN, (F)Gn
1477.726	H2N5-AA	I	Gal-LDN, LDN	2484.282	F3H3N7-AA	I	Gal-LDN(F_{0-3}), LDN(F_{0-3})

[a] Registered *m/z* for the given glycan composition when measured by MALDI in negative ion reflectron mode.
[b] F, Fucose; H, Hexose; N, *N*-acetyl-hexosamine, AA, 2-aminobenzoic acid.
[c] O-glycan core type; I, Galβ1-3(GlcNAcβ1-6)GalNAc (mucin-type) core; II, Galβ1-3(Galβ1-6)GalNAc (*S. mansoni*-specific) core; III, *S. mansoni*-specific core modified by additional β1-6Gal to Gal; IV, -3GalNAcβ1-4GlcNAcβ1-3Galα1-motif (cercarial glycocalyx structure); V, peeling product.
[d] Putative glycan motifs are proposed on the basis of interpretation of the mass spectra aided by literature (Khoo et al., 1995; Huang et al., 2001; Jang-Lee et al., 2007) and our unpublished MS/MS data; LeX, Galβ1-4(Fucα1-3)GlcNAcβ1-; LDN, GalNAcβ1-4GlcNAcβ1-; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAcβ1-; F-LDN, Fucα1-3GalNAcβ1-4GlcNAcβ1-; F-LDN-F, Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1-.

and weak binding to a few of the egg- and cercarial-derived O-glycan fractions.

Anti-F-LDN(-F) mAb 291-5D5 binds to several glycoproteins derived from cercariae and eggs as well as to glycolipids from cercariae, adult worms and eggs (Robijn et al., 2005). It binds to the excretory system of adult worms, egg shells and miracidia, and to whole cercariae (Table 2). On the array, this mAb showed binding to almost all egg and cercarial O-glycan containing HPLC fractions, indicating that many of the O-glycans contain F-LDN and/or F-LDN-F (Fig. 2D). Strong binding was also observed to many of the N- and GSL-glycans isolated from cercariae and eggs. Binding to worm-derived O- and N-glycans was not observed (Fig. 2D). Although this mAb has been described as an anti-F-LDN antibody, it also binds to F-LDN-F (Table 2) so no clear distinction can be made between structures expressing F-LDN and F-LDN-F elements on the basis of mAb 291-5D5.

3.3. Natural infection sera

The mAb binding indicates that the newly developed O-glycan microarray is effective for the detection of anti-glycan antibodies in a similar manner to the N- and GSL-glycan array, and suggest that specific, potentially different glycan epitopes are present on schistosome O-glycans. To further explore the microarray for discovering antigenic O-glycans motifs, we analysed responses in natural schistosome infections (Fig. 3). To this end we incubated the arrays with pooled sera obtained from a heavily *S. mansoni*-infected population in Uganda and compared responses from infected children (<12 years of age, Fig. 3A, B) and adults (>20 years of age, Fig. 3C, D). In these sera, large differences in responses to N- and GSL-glycans have previously been described (Van Diepen et al., 2012a) but antibody responses to O-glycans have not yet been studied. As shown previously, the overall IgG and IgM response patterns

Table 2
Specificity and binding avidity of anti-carbohydrate binding monoclonal antibodies reactive with different life stages of *Schistosom amansoni*.

		Immunofluorescence assay[a]										Glycan microarray[b]								
		Adult worm				Egg			Cercariae											
	Ig isotype	Gut	Excr. system	Parenchyma	Tegument	Shell	Miracidium	Excr. antigen	Whole	Partial	Oral sucker	AWA	AWA-TCA	SEA	ES	CA	LeX-BSA	LDN-BSA	LDN-F-BSA	F-LDN-BSA	F-LDN-F-BSA
291-4D10-A	M	+	–	+	+	+	–	+	–	–	+	+/–	+	+	+	–	+	–	–	–	–
273-3F2	M	–	–	+	+	+	+	+	–	–	–	–	–	+	+	–	–	+	–	–	–
114-4E8	G	+	–	+	+	+	+	–	–	–	–	–	–	+	+	–	–	–	+	–	–
291-5D5	M	–	+	–	–	+	+	–	+	–	–	+/–	+	+	+	+	–	–	+/–	+	+

[a] Binding specificity based on published (van Remoortere et al., 2000; Robijn et al., 2005; Wuhrer et al., 2006) and our unpublished data.
[b] AWA, adult worm antigen; AWA-TCA, TCA-precipitate of AWA; SEA, soluble egg antigen; excr. excretory; ES, excretory/secretory (glyco)proteins from eggs; CA, cercarial antigen; LeX, Galβ1-4(Fucα1-3)GlcNAcβ1-; LDN, GalNAcβ1-4GlcNAcβ1-; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAcβ1-; F-LDN, Fucα1-3GalNAcβ1-4GlcNAcβ1-; F-LDN-F, Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1-.

against the different glycan fractions in the N- and GSL-glycan microarray were similar for children and adults, but with higher fluorescence intensities in children. However, the O-glycan microarray showed more pronounced differences between children and adults, especially for IgG. There was a much higher IgG response against cercarial- and egg-derived O-glycans in children compared with adults (Fig. 3A, C), especially to the complex high mass O-glycans originating from later HILIC fractions. For IgM, the response against egg and worm O-glycans was moderate in children and absent in adults while the IgM response against cercarial O-glycans was comparable between children and adults (Fig. 3B, D).

3.4. Characterisation of differentially recognised O-glycans

To characterise some of the O-glycans to which serum antibodies bound, we focused on the most pronounced antibody responses (median fluorescence intensity >20,000). Since we used pooled sera, no statistical analysis could be applied to compare responses between children and adults for individual glycan fractions. Therefore, we applied a set of strict selection criteria to the dataset to look for the most extreme differences. The criteria for selection were such that the ratio of response between children and adults was greater than 10 and the absolute difference in fluorescence intensity was at least 10,000. This resulted in seven differentially recognised egg-derived and 18 cercarial-derived O-glycan fractions for IgG (Table 3) and none for IgM. The glycan structures present in these differentially recognised fractions were analysed by MALDI-TOF-MS (Table 3 and Supplementary Table S1). Most of the fractions contained mixtures of glycans, mainly due to variations in fucose contents. For the differentially recognised cercariae-derived O-glycans, the majority of glycan compositions have a relatively high *N*-acetyl-hexosamine content with a variable number of fucose residues (indicating the presence of fucosylated Galβ1-4GalNAcβ1-4GlcNAc or GalNAcβ1-4GlcNAcβ1-3Gal motifs as previously described (Khoo et al., 1995; Jang-Lee et al., 2007)). To confirm this, we performed MALDI-TOF-MS/MS analysis on cercarial O-glycan fraction 15.6 which contained five related glycans composed of identical H_3N_6 core structures but substituted with different numbers of fucose residues. The fragmentation spectra suggest that these glycans were composed of GalNAcβ1-4GlcNAcβ1-3Gal repeats which showed clear differences in fucose substitution patterns (Fig. 4). The glycan masses detected at *m*/*z* 2865.1 and 3157.2 $[M-H]^-$ were the most abundant (Fig. 4 total spectrum), and the MS/MS analysis (Fig. 4C, E) indicated that both contain the terminal Fucα1-2Fucα1-3GalNAc(Fucα1-2Fucα1-2Fucα1-3)GlcNAc motif. We also showed that fucosylated H_4N_8 containing glycan fractions such as $F_{10}H_4N_8$ in cercarial O-glycan fraction 18.5 were composed of GalNAcβ1-4GlcNAcβ1-3Gal units (Supplementary Fig. S1). Cercarial O-glycan fraction 15.1 contains two types of glycan structures which are unique to schistosome O-glycans. This fraction also contains H_4N_8 with up to four fucoses, but its major component is $F_2H_6N_3$ which can only be translated into the *S. mansoni*-specific structure with a core GalNAc that has two branches composed of Galβ1-4(Fucα1-3)GlcNAcβ1-3(Galβ1-6)Galβ1. This glycan has been described as being found specifically in cercarial O-glycans (Jang-Lee et al., 2007).

The majority of egg-derived O-glycan fractions that showed differential binding of IgG in the children versus adults comparison have a more equal share of *N*-acetyl-hexosamines and hexoses which suggests the presence of O-glycans based on Gal-GlcNAc or LeX repeats (Jang-Lee et al., 2007). Fraction 32.2 is a fraction that contains glycans with different basic compositions. MALDI-TOF-MS/MS analysis of each component indicated that most structures indeed contain LeX (Supplementary Fig. S1). In addition to MS, incubation with defined mAbs aided the annotation of the

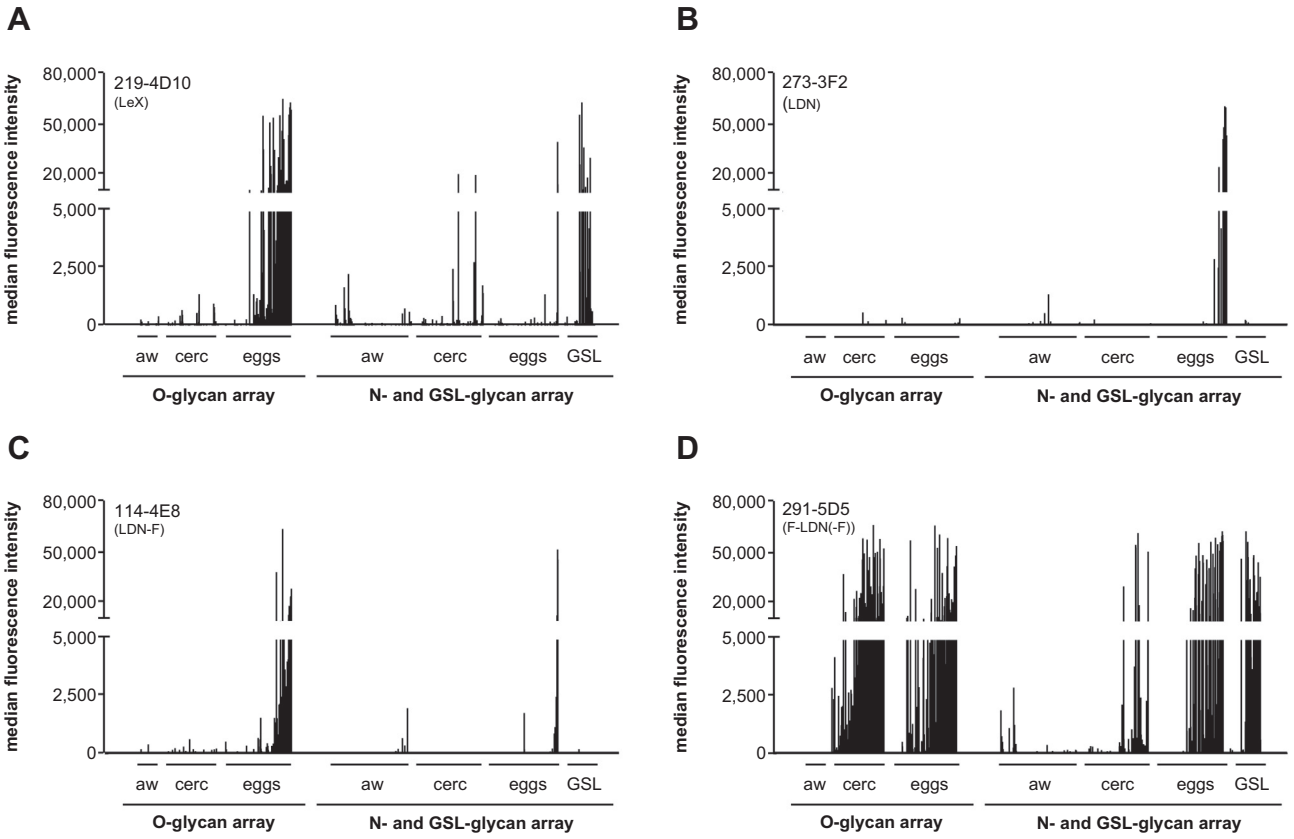

Fig. 2. Binding of monoclonal antibodies (mAbs) to O-glycans, N-glycans and glycosphingolipid-glycans isolated from different life stages of *Schistosoma mansoni*. Log_2 background subtracted median fluorescence intensities are shown for 291-4D10 (A, binds to LeX), 273-3F2 (B, binds to LDN), 114-4E8 (C, binds to LDN-F), and 291-5D5 (D, binds to F-LDN(-F)). Aw, adult worms; cerc, cercariae; GSL, cercarial glycoshingolipid glycans.

glycan elements and glycan structures present in the printed fractions (Table 3).

4. Discussion

Over the last few years significant progress has been made in characterising the antigenic properties of N-glycans of *S. mansoni* and other parasitic helminths. In contrast, much less is understood about O-glycans and antibody responses to these have not yet been systematically analysed. We have generated the first known shotgun O-glycan microarray containing O-glycans isolated from *S. mansoni* cercariae, adult worms and eggs, allowing the study of antibody responses to naturally occurring O-glycans. Incubations with natural infection sera showed highly abundant responses to O-glycans, underlining the importance of the inclusion of these often schistosome-specific O-glycans in glycan antigen studies. As shown previously by glycan microarray analysis (Van Diepen et al., 2012a), IgG and IgM responses to N-glycans and GSL-glycans were higher in schistosome-infected children compared with adults (Fig. 2) in a Ugandan population. Since these child and adult pools were matched for infection intensity and balanced for sex, these results are likely due to a phenomenon that reflects human host age or duration of infection in schistosomiasis-endemic areas. Using the new O-glycan microarray, more pronounced differences were observed between the children and adult groups. In particular, IgG responses against relatively large and more complex cercarial and egg-derived O-glycans were higher in children. Similar to IgM responses against N-glycans and GSL-glycans, the IgM response against egg-derived O-glycans was higher in children that in adults. However, the response against cercarial-derived O-glycans was found to be comparable between children and adults, both with respect to fluorescence intensity and to glycan targets bound. This is an interesting observation which warrants further research to determine what causes the relatively high and specific anti-cercarial O-glycan IgM responses in the adult group, while anti-egg O-glycan responses were decreased similar to all of the other anti-glycan responses in the adult group. One possible explanation is that while adults in general decrease overall antibody responses to schistosome glycans, exposure to new invading cercariae still leads to the induction of strong antibody responses to those O-glycans that are unique to those larvae. These observations in any case illustrate that schistosome O-glycans contain antigenic glycan motifs that are a relevant addition to those present on N-glycans and GSL-glycans.

During the process of isolation and fractionation of O-glycans for construction of the shotgun O-glycan microarray, we were able to characterise a large set of O-glycans expressed by *S. mansoni* using MALDI-TOF-MS. The O-glycosylation profiles of eggs and cercariae were found to be largely in line with those reported by Jang-Lee et al. (2007) and showed life stage-specific glycan compositions as well as shared glycan elements. The majority of cercarial O-glycans contain a Galβ1-3(Galβ1-6)GalNAc (*Schistosoma*-specific) core which can be modified by additional β1-6Gal residue, while the Galβ1-3(GlcNAcβ1-6)GalNAc (mucin type-2) core is the most predominant in egg-derived O-glycans (Khoo et al., 1997; Jang-Lee et al., 2007). The modified hydrazinolysis method that we used allows the release of O-glycans with free reducing termini in high yields with reduced levels of degradation products (Kozak et al., 2012). Indeed, we were able to isolate many different intact O-glycans in amounts which allowed the construction of a microarray by which antibodies to O-glycans could be sensitively detected. In some of the selected cercarial O-glycan containing

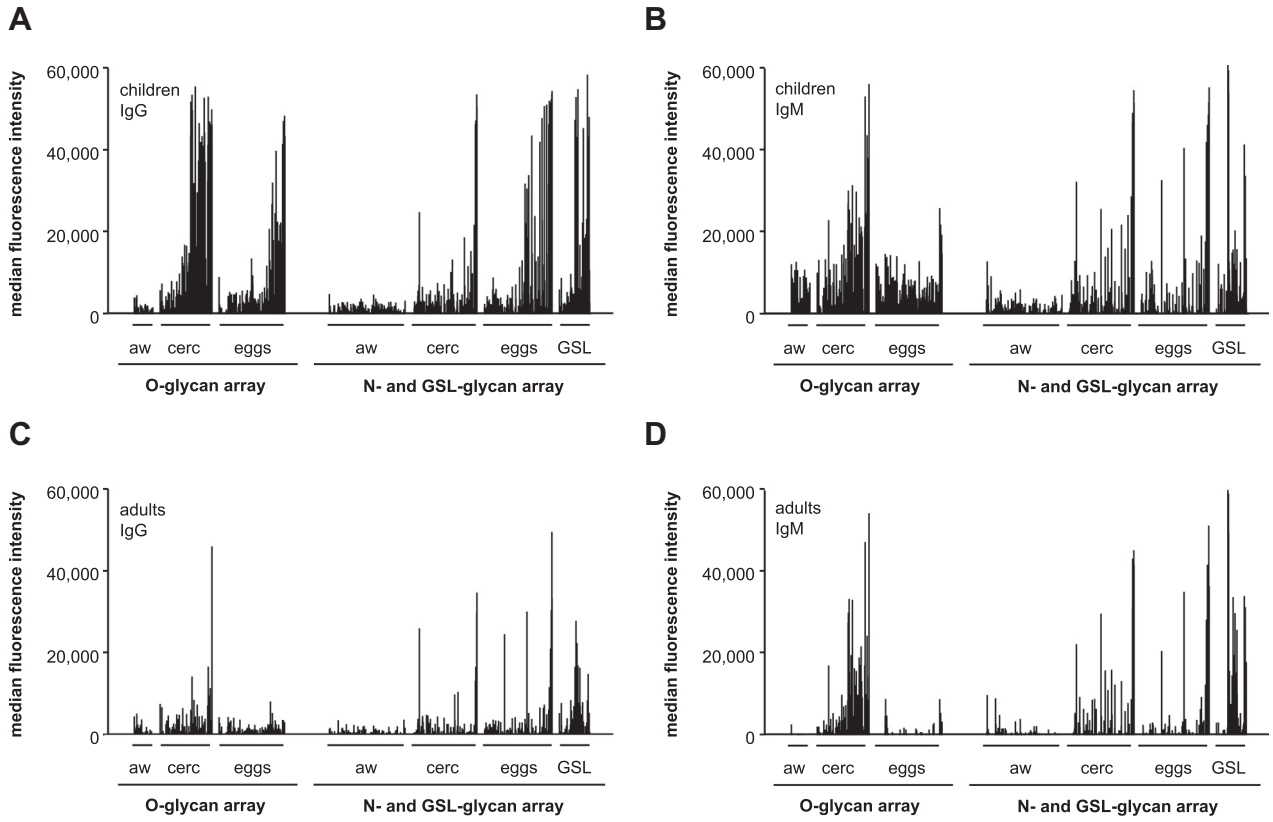

Fig. 3. Binding of serum antibodies from *Schistosoma mansoni*-infected children <12 years (A, B) and adults >20 years of age (C, D) to glycans from different life stages of *S. mansoni*. Average background subtracted median fluorescence intensities are shown for IgG (A, C) and IgM (B, D). Aw, adult worms; cerc, cercariae; GSL, cercarial glycoshingolipid glycans.

fractions, however, we detected masses corresponding to H_3N_6 or H_4N_8 with different degrees of fucosylation (Table 3). MALDI-TOF-MS/MS revealed linear structures, as shown in Fig. 4 and Supplementary Fig. S1, which appeared to be peeling products of large glycocalyx O-glycans, reflecting only one branch of the original structure (Khoo et al., 1995). Such degradation products were previously unexpectedly observed during conventional hydrazinolysis release of core-2 based multifucosylated LDN containing schistosome egg O-glycans (Robijn et al., 2007). Apparently glycocalyx-derived O-glycans are very sensitive to partial degradation, even during the modified hydrazinolysis procedure applied (Kozak et al., 2012). This partial degradation still allowed 2-AA-reaction of the reducing end monosaccharide and did not limit the goal of our study. The antigenic glycan elements present within the linear branch derived from the degraded branched core glycan are intact and strong antibody responses were observed (Fig. 3; Table 3). In addition to MS, incubation with defined mAbs enabled us to increase our knowledge of the glycan elements and glycan structures in the fractions printed on the O-glycan array (Table 3). This is a vital step towards a better understanding of the specificity of the anti-glycan antibodies induced by infection with schistosomes. Due to the extreme complexity of the schistosome O-glycome, even a two-dimensional HPLC separation of O-glycans did not result in a complete separation of all glycans present. However, the array does allow the definition of a number of antigenic motifs that were not previously identified. An extensive list of glycan compositions was composed for both egg and cercarial O-glycans through MALDI-TOF-MS analysis and mAb incubations (Tables 1 and 3), but we were unable to detect any glycan compositions in the O-glycans released from adult worms. This is in line with an earlier study in which no O-glycans were detected in a chemically released glycan pool obtained from adult worms (Wuhrer et al., 2006). A possible explanation for the absence of detectable O-glycans in these adult worm glycan pools is that they may be present in amounts that are too low to be measured without further concentration and purification. The well-described gut-associated antigens, CCA and CAA, that carry long polymeric O-linked carbohydrates chains, were also not detected (Bergwerff et al., 1994; van Dam et al., 1994). After fractionation by HILIC and RP-HPLC we were able to detect a few O-glycans, albeit only in very small amounts which suggests that, besides expression on CAA and CCA, O-linked glycosylation is limited on *S. mansoni* adult worm glycoproteins. In four of the isolated RP-HPLC fractions we were able to detect glycan masses corresponding to structures containing three hexuronic acid (HexA) and three HexNAc residues ($HexA_3HexNAc_3$-AA) which is a composition that is characteristic for CAA which carry glucuronic acid-substituted GalNAc polymers (Bergwerff et al., 1994).

In addition to providing structural information on O-glycans on the array, incubations with mAbs also revealed more detailed glycan specificity of the mAbs and consequently about the glycans visualised in IFA at the surface of the different schistosome life stages (Table 2). For instance mAb 291-4D10-A, which has specificity for synthetic LeX, showed binding to SEA and O-glycans derived from SEA but not to CA nor to the majority of cercarial O- and N-glycans. LeX is, however, expressed by cercarial N- and O-glycans (Huang et al., 2001; Khoo et al., 2001; Jang-Lee et al., 2007; Van Diepen et al., 2012b), but either the relative expression levels are insignificant in the released glycan sample due to relatively high abundance of fucosylated GalNAcβ1-4GlcNAcβ1-3Gal variants in the cercarial O-glycan fractions (Fig. 4) and therefore less fractions contain LeX, or the mAb has a specificity for a particular structural context of LeX that is associated with the egg but not the cercarial glycans. Possibly, binding of the mAb to the

Table 3
Differential IgG responses to O-glycans in children <12 years of age and adults >20 years.

	MFI[a]		Monoclonal antibody binding[b]				Glycans present in fraction		
	Children	Adults	291-4D10 (LeX)	273-3F2 (LDN)	114-4E8 (LDN-F)	291-5D5 (F-LDN(-F))	Composition[c]	Core[d]	Proposed glycan motif(s)[e]
32.2 SEA	40,359	3366	+++	–	+	+++	$F_{2\text{-}3}H_4N_4$	I,II	LeX, di-LeX, tri-LeX, LN, (F)Gn
							$F_3H_5N_4$	II	LeX, di-LeX, tri-LeX
							$F_{3\text{-}4}H_3N_5$	I	Gal-LDN($F_{0\text{-}4}$)
							$F_{2\text{-}4}H_4N_5$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
32.4 SEA	22,962	1096	+++	–	++	++	$F_{2\text{-}3}H_4N_4$	I,II	LeX, di-LeX, tri-LeX, LN, (F)Gn
							H_9N_2	V	Mannose
							$F_{2\text{-}4}H_3N_5$	I	Gal-LDN($F_{0\text{-}4}$)
33.3 SEA	22,315	2133	++	+/–	+	++	$F_{2\text{-}4}H_4N_5$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
							$F_{2\text{-}4}H_3N_6$	I	Gal-LDN($F_{0\text{-}4}$), (F)Gn
34 SEA	22,841	2194	++	–	+	++	$F_{3\text{-}4}H_5N_4$	II	LeX, di-LeX, tri-LeX
							$F_{2\text{-}4}H_4N_6$	I	(F)Gn
							$F_{3\text{-}4}H_3N_7$	I	Gal-LDN($0_{3\text{-}4}$), LDN($F_{0\text{-}4}$), (F)Gn
36.2 SEA	34,861	3303	+++	–	+	++	$F_{3\text{-}4}H_5N_5$	I,II	LeX, di-LeX, tri-LeX, LN, (F)Gn
							$F_4H_4N_6$	I	(F)Gn
							$F_{4\text{-}5}H_5N_6$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
							$F_{4\text{-}5}H_6N_5$	II	LeX, di-LeX, tri-LeX
36.3 SEA	39,196	3364	+++	–	++	+++	$F_{3\text{-}4}H_4N_6$	I	(F)Gn
							$F_4H_5N_5$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
							$F_{3\text{-}5}H_5N_6$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
							$F_{3\text{-}5}H_4N_7$	I	Gal-LDN($F_{0\text{-}5}$)
36.5 SEA	48,911	3928	+++	+/–	++	+++	$F_4H_4N_6$	I	(F)Gn
							$F_4H_5N_5$	I,II	LeX, di-LeX, tri-LeX, (F)Gn
10.6 CA	44,023	3682	–	–	–	+++	$F_{2\text{-}5}H_1N_4$	I	LDN($F_{0\text{-}5}$), $F_{0\text{-}3}$Gn
							$F_{0\text{-}2}H_4N_3$	II	LeX, di-LeX, LN
10.10 CA	50,204	3649	–	–	–	++	H_6N_2	V	Mannose
							$F_{1\text{-}6}H_2N_4$	IV	LDN($F_{0\text{-}6}$)
							$F_{1\text{-}6}H_2N_5$	I	LDN($F_{0\text{-}6}$), Gal-LDN($F_{0\text{-}5}$)
11.5 CA	56,047	5251	–	–	–	+++	$F_{2\text{-}7}H_2N_4$	IV	LDN($F_{1\text{-}6}$)
12.7 CA	37,977	2057	–	–	–	++	$F_{5\text{-}8}H_2N_5$	I	LDN($F_{0\text{-}6}$), Gal-LDN($F_{0\text{-}5}$)
12.8 CA	28,324	2031	–	–	–	++	H_7N_2	V	mannose
							H_8N_2	V	mannose
13.4 CA	31,722	2218	–	–	–	+	$F_{1,2,4}H_4N_8$	IV	LDN($F_{0\text{-}4}$)
14 CA	42,474	2478	–	–	–	++	$F_2H_5N_3$	III	LeX, FGn
							$F_3H_5N_4$	II,III	LeX, di-LeX, tri-LeX, FGn
15.1 CA	21,256	1898	–	–	–	++	$F_{1\text{-}2}H_6N_3$	III	LeX, LN
							$F_{0\text{-}4}H_4N_8$	IV	LDN($F_{0\text{-}4}$)
15.2 CA	39,693	2962	–	–	–	++	$F_{0\text{-}5}H_4N_8$	IV	LDN($F_{0\text{-}5}$)
							$F_2H_6N_3$	III	LeX
							$F_{1\text{-}2}H_6N_4$	II,III	LeX, di-LeX, LN, (F)Gn
							$F_{4\text{-}7,9}H_3N_7$	I	LDN($F_{0\text{-}5}$), Gal-LDN($F_{0\text{-}5}$)
15.5 CA	38,916	2465	–	–	–	+++	$F_{2\text{-}8}H_3N_6$	IV	LDN($F_{0\text{-}6}$)
							$F_{2,4}H_4N_8$	IV	LDN($F_{0\text{-}4}$)
15.6 CA	53,281	3961	–	–	–	++	$F_{5\text{-}9}H_3N_6$	IV	LDN($F_{0\text{-}6}$)
15.8 CA	44,816	3124	–	–	–	++	H_9N_2	V	mannose
							$F_{4\text{-}6}H_3N_7$	I	LDN($F_{0\text{-}6}$), Gal-LDN($F_{0\text{-}5}$)
17.3 CA	41,735	3941	+/–	–	–	++	$F_2H_7N_4$	II	LeX, di-LeX, LN
18.5 CA	47,495	3439	–	–	–	+	$F_{6\text{-}12}H_4N_8$	I	LDN($F_{0\text{-}6}$)
20.2 CA	36,878	2781	–	–	–	++	$F_{4\text{-}6}H_2N_5$	I	LDN($F_{0\text{-}6}$), Gal-LDN($F_{0\text{-}5}$)
21 CA	50,473	4510	–	–	–	++	nd	–	–
22 CA	31,962	3094	–	–	–	++	nd	–	–

SEA, soluble egg antigen; CA, cercarial antigen.

[a] MFI, median fluorescence intensity.

[b] Binding intensity factor compared with background signal (-, no binding; +/–, factor >2; +, factor >5 ++, factor >25; +++, factor >100).

[c] F, Fucose; H, Hexose; N, *N*-acetyl-hexosamine.

[d] O-glycan core type; I, Galβ1-3(GlcNAcβ1-6)GalNAc (mucin-type) core; II, Galβ1-3(Galβ1-6)GalNAc (*Schistosoma mansoni*-specific) core; III, *S. mansoni*-specific core modified by additional β1-6Gal to Gal; IV, -3GalNAcβ1-4GlcNAcβ1-3Galα1-motif (cercarial glycocalyx structure); V, N-glycan contaminant.

[e] Putative glycan motifs are proposed on the basis of interpretation of the mass spectra aided by literature (Khoo et al., 1995; Huang et al., 2001; Jang-Lee et al., 2007) and our unpublished MS/MS data; LeX, Galβ1-4(Fucα1-3)GlcNAcβ1-; LDN, GalNAcβ1-4GlcNAcβ1-; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAcβ1-; F-LDN, Fucα1-3GalNAcβ1-4GlcNAcβ1-; F-LDN-F, Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1-.

LeX element is hindered by expression of the Galβ1-3(Galβ1-6)GalNAc core which is found in cercariae (Huang et al., 2001; Jang-Lee et al., 2007). Binding was, however, observed against cercarial glycolipid glycans present on the N- and GSL-glycan array. This is in line with a study by Robijn et al. (2005) where binding of this antibody was mainly observed to cercarial glycolipid glycans and egg glycoproteins. Since we observed hardly any binding to egg-derived N-glycans but very abundant binding to egg-derived O-glycans in our glycan microarray analysis we can conclude that this antibody, 291-4D10-A, mainly bound to egg glycoproteins expressing O-linked glycans.

In conclusion, we have succeeded in generating the first known *S. mansoni* O-glycan microarray suitable for studying antibody responses to a type of glycan which has to date been mostly ignored. Incubations of mAbs and infection sera with the O-glycan microarrays showed that O-glycans provide specific antigen targets. Antibodies to O-glycans in *S. mansoni* infection sera are abundant and therefore likely play a role in immunity to *Schistosoma*.

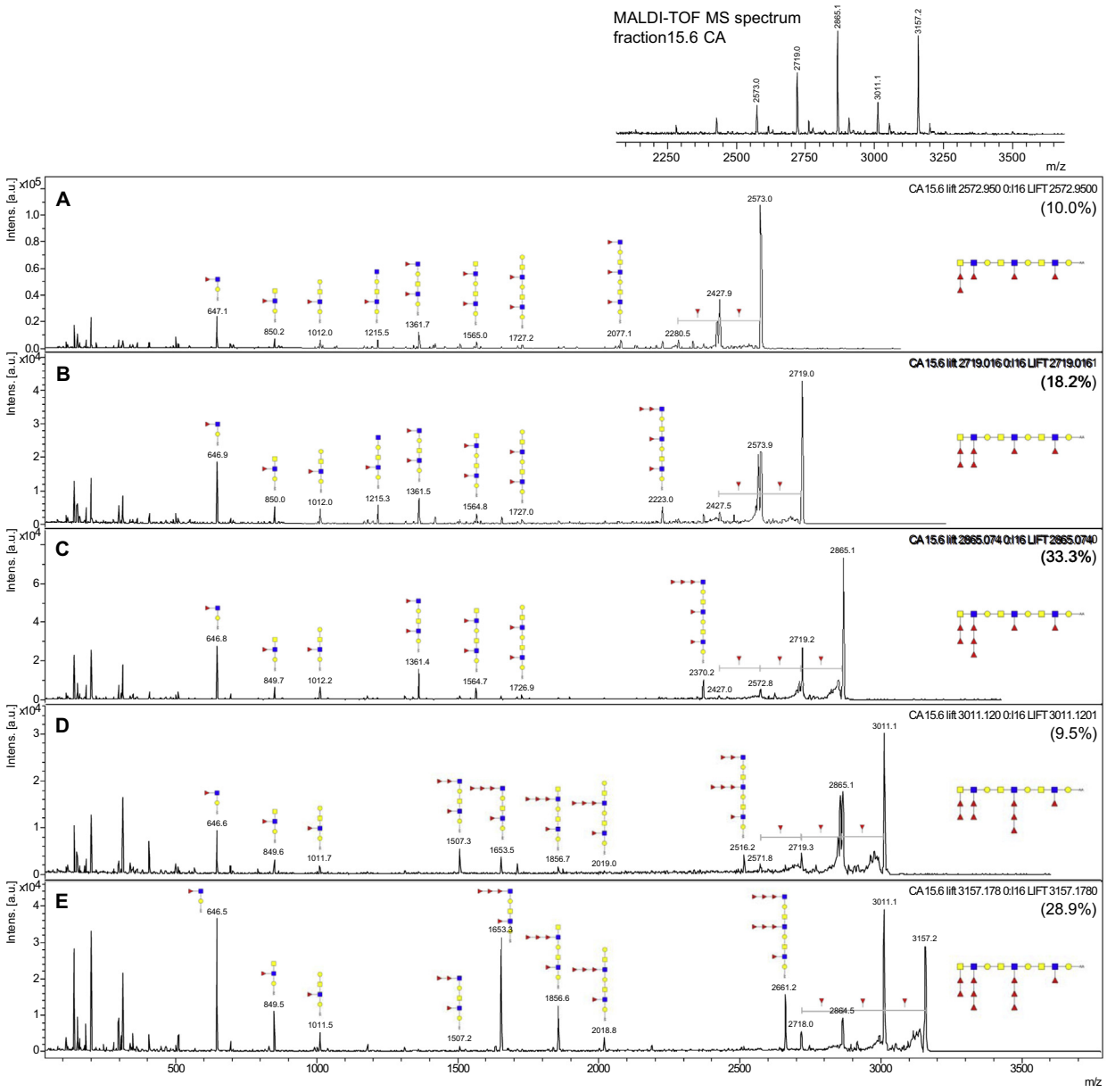

Fig. 4. MALDI-TOF-MS/MS spectra of glycans detected in *Schistosoma mansoni* cercarial antigen (CA) fraction 15.6 at *m/z* 2573.0 $[M-H]^-$ (A), 2719.0 $[M-H]^-$ (B), 2865.1 $[M-H]^-$ (C), 3011.1 $[M-H]^-$, and 3157.2 $[M-H]^-$ (E). Blue square, *N*-acetylglucosamine; yellow square, *N*-acetylgalactosamine; yellow circle, galactose; red triangle, fucose. The percentage of each mass within cercarial antigen fraction 15.6 was calculated based on peak intensities in the MALDI-TOF analysis and is depicted in parentheses above the structure. Y axis shows peak intensity (Intens.) in arbitrary units (a.u.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Further studies are necessary to understand how anti-O-glycan antibodies are generated, both for those that cross-react with N- and GSL-glycans and for those that are uniquely directed against O-glycan motifs.

The isolation of N-glycans, O-glycans and GSL-glycans, and the generation of a shotgun glycan microarray as described for *S. mansoni* in this study, can be applied to all other helminth parasites that express antigenic glycans. When printed on a glass slide, these glycans can now cover the full range of naturally occurring protein- and lipid-linked glycans and glycan elements of each species studied. Such glycan microarrays will be invaluable tools in studies of anti-glycan antibody profiles and dynamics in human and animal helminthiases. These studies will aid the search for glycan vaccine candidates and glycan antigens for diagnostic antibody detection assays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.02.008.

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